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Regulation of Vitellogenesis in the Fiddler Crab, *Uca pugilator*

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Abstract. Protein synthesis was measured in ovary and hepatopancreas of intact and eyestalk-ablated fiddler crabs (*Uca pugilator*) *in vitro*. A crude extract of eyestalks from the shrimp *Penaeus setiferus* inhibited ovarian weight gain in eyestalk-ablated crabs. This crude eyestalk extract also inhibited *in vitro* protein synthesis in ovaries from intact and eyestalk-ablated crabs. A polyclonal antibody to crab vitellogenin was used to measure vitellogenin synthesis in ovaries, hepatopancreas, and hemolymph *in vitro*. Gonad-inhibiting hormone was partially purified from the crude eyestalk extract. The partially purified material inhibited vitellogenin synthesis in ovarian tissues *in vitro*.

Introduction

Panouse (1943) first observed accelerated gonadal growth in eyestalk-ablated shrimp, *Palaemon serratus*. Eyestalk ablation induces precocious gonadal development in almost all crustaceans (Charniaux-Cotton, 1985; Quackenbush, 1986; Fingerman, 1987). The endocrine nature of this response was confirmed by the implantation of sinus glands into eyestalk-ablated fiddler crabs (*Uca pugilator*). The implanted neurohemal organs suppressed gonadal development in eyestalk-ablated crabs (Brown and Jones, 1948). The rapid ovarian development that occurs after eyestalk ablation was attributed to the absence of a gonad-inhibiting hormone (GIH). The chemical characteristics of partially purified GIH resembles other peptide hormones produced in the eyestalk neurosecretory system (Charniaux-Cotton, 1985). GIH has not yet been fully characterized due in part to the difficulty in the bioassay of an inhibitory hormone (Channing *et al.*, 1985).

The ovaries and testes were accepted as the target tissues of GIH because they both develop rapidly after eyestalk ablation. Crude eyestalk extracts directly inhibit ovarian protein synthesis when tested *in vitro* (Gorell and Gilbert, 1971; Eastman-Reks and Fingerman, 1984). These reports confirmed that the eyestalk neuroendocrine system can directly suppress gonadal development in crustaceans. The primary product of ovarian protein synthesis is the egg yolk protein vitellin (Vn) (Eastman-Reks and Fingerman, 1984; Lui and O'Connor, 1976). However, it was generally accepted that the precursor to Vn, vitellogenin (Vg) was made in extra-ovarian tissues in crustaceans (Wallace *et al.*, 1967; Fielder *et al.*, 1971; Charniaux-Cotton, 1985). The demonstration that isolated ovaries synthesized Vg challenged the significance of extra-ovarian Vg synthesis (Lui and O'Connor, 1977). Both the hemocytes of the hemolymph and the hepatopancreas of crustaceans are capable of Vg synthesis and therefore also targets for GIH (Kerr, 1969; Souty and Picaud, 1984). The crustacean hepatopancreas synthesizes digestive enzymes and hemocyanin and may also produce Vg (Gibson and Barker, 1979; Senkbeil and Wriston, 1981; Paulus and Laufer, 1987; Tom *et al.*, 1987). An understanding of the relative contributions by the potential tissue sources of Vg is important to the development of a valid GIH bioassay.

This study was undertaken to determine if the hepatopancreas, hemolymph, and the ovaries of the fiddler crab (*Uca pugilator*) produced Vg. *In vitro* production of Vg was then used as a specific bioassay for the characterization and purification of GIH.

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Abbreviations: ESE, eyestalk equivalents; GIH, gonad inhibiting hormone; HEPES, N-2-Hydroxyethylpiperazine-N-2-ethane sulfonic acid; IgG, immunoglobulin; mOS, milliosmoles; PBS, phosphate buffered saline; Rf, retention factor; Vg, vitellogenin; Vn, vitellin.

Materials and Methods

Animals

Fiddler crabs (*Uca pugilator*) were purchased monthly from Gulf Specimen Co. (Panacea, Florida). Crabs were maintained in a recirculating seawater aquarium (25‰; 24°C) and fed daily with oatmeal. Crabs were eyestalk-ablated with fine scissors; the cut stumps were cauterized. To obtain ovaries with uniform oocyte diameters, the ovaries were dissected from crabs 8 days after eyestalk ablation. The average oocyte diameters from these animals were quite consistent (0.125 ± 0.001 mm; n = 60). The oocyte diameters of eggs just released and attached to pleopods of female crabs were 0.25 ± 0.01 mm (n = 90). Based on their size, the oocytes of crabs 8 days after eyestalk ablation were about halfway through secondary vitellogenesis. The ovaries from these ablated crabs were a characteristic deep purple color of vitellogenic fiddler crab eggs (Brown and Jones, 1948; Webb, 1977; Eastman-Reks and Fingerman, 1984). For all subsequent assays, ovaries were obtained from crabs 8 days after eyestalk ablation. Eyestalk-intact crabs provided ovarian tissues for controls, and the oocyte diameters from these animals were also recorded.

Eyestalk extract

Eyestalks from adult (>30 g) male and female *Penaeus setiferus* were obtained frozen from a commercial shrimp processor (Coastal Freezing, Port Aransas, Texas). One hundred eyestalks (22.25 g) were ground frozen in a mortar and pestle and extracted in distilled water. The extract was gently boiled for 5 min and then centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant (2 eyestalk equivalents/ml) was stored frozen at −4°C.

In vivo *GIH bioassay*

Crabs from the stock tank were eyestalk-ablated as described. One group (n = 10) had their ovaries removed and were weighed to the nearest 0.1 mg at the start of the experiment. Five other groups (all n = 10) were injected on alternate days for 15 days as described by Quackenbush and Herrnkind (1983). Four different doses of crude eyestalk extract and a saline control were tested for effects on ovarian weight gain after eyestalk ablation (Cooke *et al.*, 1977).

In vivo *^{14}C leucine incorporation*

^{14}C leucine (270 μCi/ml; ICN Radiochemicals, Irvine, California) was diluted with cold leucine (0.127 *M*) to achieve a final dose of leucine of 5 nmole/0.5 μCi/10 μl. Both intact and eyestalk-ablated crabs were injected with 10 μl of the stock ^{14}C leucine (5 nmole/0.5 μCi/10 μl) and held in dry containers after injection. Four hours or 24 hours after ^{14}C leucine injection a sample of hemolymph (50 μl) was removed from the crabs (n = 10; 2 replicates) and then added to small tubes containing 450 μl of extraction buffer (0.5 *M* NaCl; 0.5 *M* Tris; 5 mM EDTA; pH 7.0, Lui and O'Connor, 1976). The ovaries and the hepatopancreas from the crabs were then removed and separately homogenized in extraction buffer (n = 10; 2 replicates). Proteins from these extracts and the hemolymph samples were precipitated by the addition of three volumes of ice cold 100% $(NH_4)_2SO_4$, followed by centrifugation at 8000 × *g* for 5 min at 4°C. The resulting pellet of protein was resuspended and precipitated as described above twice more. The final pellets of protein were dissolved in phosphate-buffered saline (PBS, Quackenbush and Fingerman, 1985). Aliquots (100 μl) of the solubilized proteins were counted for ^{14}C leucine incorporation with a commercial scintillation fluor using liquid scintillation spectrometry. Counts per minute (CPM) were converted to disintegrations per minute (DPM) via an internally stored quench curve. Total protein of these samples was measured using the Bio-Rad Protein Reagent assay (Bio-Rad, Richmond, California) Bovine serum albumin (1 mg/ml) was used as a standard. Results from this assay are expressed as DPM/mg protein based on these determinations. Differences between treatment groups were tested using a standard *t*-test (Sokal and Rohlf, 1969).

In vitro *^{14}C leucine incorporation*

Tissues (ovary, hepatopancreas, or muscle) were removed from crabs by dissection under chilled saline. Tissue fragments (3–6 mm) were rinsed with saline and placed into a tissue culture media (n = 12; 2 replicates) (Reddy and Wyatt, 1967). The culture media consisted of amino acids, 0.066 *M* glucose, 0.033 *M* trehalose, 5 m*M* HEPES, and 0.001% ampicillin in crustacean saline (pH 8.2; 1100 mOS, Cooke *et al.*, 1977). Prior to use the media was filter sterilized (Nalgene, 20 μ filter). Eyestalk extracts (100 μl) or muscle extracts (100 μl) were added to the incubation media (2000 μl) that contained the crab tissue fragments. Finally, ^{14}C leucine (5 nmole/0.5 μCi/10 μl) was added to the incubation media. Beakers containing the media and tissue fragments were placed in a sealed chamber that was gassed with 95% O_2/5% CO_2 at 4 psi. During a 4-h incubation at 30°C, the beakers were gently agitated on an orbital shaker. At the termination of the incubation, tissues were removed from the media, rinsed with chilled saline, and then homogenized in ice cold extraction buffer. The tissue homogenates were centrifuged at 8000 × *g* for 15 min at 4°C. Proteins in the supernatant were precipitated by the addition of 3 vol-

umes of ice cold 100% $(NH_4)_2SO_4$ and then recentrifuged at 8000 × *g* for 15 minutes at 4°C. The pellet of protein was resuspended in extraction buffer, and the entire precipitation procedure was repeated twice more. The final pellet of protein was resuspended in PBS. Aliquots (100 μl) of the solubilized proteins were taken for the determination of both total protein and total ^{14}C leucine incorporation as described above. Differences between treatment groups were tested using a *t*-test (Sokal and Rohlf, 1969).

Time course of in vitro *^{14}C leucine incorporation*

Tissues were dissected (n = 12; 2 replicates) and incubated for 2, 4, 8, 16, or 22 hours as described above. At the termination of incubation, the tissues were rinsed with chilled crustacean saline, homogenized in 0.4 *N* perchloric acid and centrifuged at 2000 × *g* for 10 minutes at 22°C. The resulting pellet of protein was resuspended in 90% ethanol/1% sodium acetate and centrifuged as above. The resulting pellet of protein was retained and extracted in chloroform:methanol (2:1) and centrifuged as above. The final pellet of protein was air dried at 50°C and then dissolved in 1 *N* NaOH. Aliquots (100 μl) of the solubilized proteins were taken for total protein determination and total ^{14}C leucine incorporation as described above. Bovine serum albumin in 1 *N* NaOH served as the standard for the protein determinations. Differences between groups were tested with a *t*-test (Sokal and Rohlf, 1969).

Vg purification and antibody production

Uca pugilator vitellogenin was characterized as a large purple lipoprotein with two subunits of 100,600 and 125,000 daltons. This protein was about 90% of the total protein in crab ovaries (Eastman-Reks and Fingerman, 1984). We used a slight modification of the Eastman-Reks and Fingerman (1984) method to isolate vitellogenin from *Uca pugilator*. Ovaries from crabs (0.10 to 0.25 mm oocyte diameter) were pooled and homogenized in ice cold extraction buffer. Phenylmethonyl sulfonyl flouride (0.001%) was added to the extraction buffer just before use to inhibit general proteases. Ovarian proteins were extracted as above using 3 cycles of extraction buffer and ice cold 100% $(NH_4)_2SO_4$ precipitation. The final protein pellet was resuspended in PBS (2 mg/ml) and applied to a Sephadex G-200 column (2.5 cm × 20 cm; void volume = 18 ml with blue dextran). The protein extract retained the purple color characteristic of the crab ovaries and eggs. This characteristic color aided us in the purification procedure. The column was eluted with PBS (4 ml/h) at 4°C, 1-ml fractions were collected. Column fractions were monitored at 280 nm, and fractions with the characteristic purple color of crab ovaries were pooled and dried on a rotary evaporator (Speed-Vac, Savant Instruments, Hicksville, New York). Samples of this crude ovarian protein extract were characterized on 7% polyacrylamide gels. Sodium dodecyl sulfate was added to the protein sample and the polyacrylamide to dissassociate the large proteins into subunits (Hames and Rickwood, 1981). The crude extract was dominated by 2 bands of protein in the size range of vitellogenin characterized by Eastman-Reks and Fingerman, 1984. A preparative gel procedure was used to purify enough of the crude material from crab ovaries for rabbit immunization. Ovarian protein samples from the G-200 column were resuspended in a dissassociating buffer with sodium dodecyl sulfate. This was applied to a large preparative gel of 7% polyacrylamide (1.0 × 150 × 130 mm) in a single well which spanned the entire width of the gel (Hames and Rickwood, 1981, Laemmli, 1970). After the tracking dye eluted from the gel, a single vertical slice of the gel was removed and stained for proteins with 0.5% Coomassie Blue to determine mobilities of the proteins within the preparative gel. The remainder of the preparative gel was sliced horizontally (1-mm segments); the resulting gel segments were placed in an elution buffer of 0.05 m*M* ammonium acetate. Proteins were eluted from the gels into the buffer at 4°C for 24 hours. Proteins that eluted from the gels were freeze dried and stored at −4°C. Samples of the proteins eluted from the gel segments were characterized on another analytical 7% polyacrylamide gel with sodium dodecyl sulfate buffers. This procedure allowed us to obtain sufficient quantities of partially purified Vg for immunization procedures. The molecular weight of the Vg subunits were determined by comparison to standards simultaneously separated in analytical polyacrylamide gels of various percentages. Molecular weight standards were obtained from Bio Rad and used according to the instructions provided. Myosin (200,000), beta-galactosidase (116,250), phosphorylase-B (97,400), bovine serum albumin (66,200), and ovalbumin (42,699) were the protein standards used to calibrate the subunit molecular weight of ovarian proteins.

Serum was obtained from rabbits before immunization via ear vein puncture. Rabbits were immunized with 50 subepidermal injections (20 μl each) of complete adjuvant containing 40 μg egg yolk protein/ml as described previously (Quackenbush and Fingerman, 1985). Rabbits were bled each week via cardiac puncture. Whole blood was allowed to clot then centrifuged at 10,000 × *g* for 30 minutes at 4°C. The supernatant serum was stored at −72°C. Serum was screened against partially purified egg yolk proteins using Ouchterlony plates (Ouchterlony, 1949). Serum showing a precipitation line against partially purified egg yolk proteins was further purified. Positive serum was passed through a column of DEAE-Affi-Gel-Blue (Bio-Rad) to separate the IgG fraction

from other serum components. A column (14 ml bed volume) was prepared and used following methods described in Bio Rad [illegible] n 1062 (Bio Rad). Fractions containing IgG [illegible] pooled, aliquoted (0.5 mg/ml), and dried on a rotary evaporator. The antibody stocks were diluted with PBS containing 0.01% sodium azide for use in the assays.

Antibody characterization

Rabbits were immunized with either the high molecular weight Vg subunit (V_1) or the low molecular weight subunit (V_2). Since the hepatopancreas samples contained only V_2, only the antibodies against V_2 were characterized. Antibody characterization included Ouchterlony plates, immunoprecipitation, and western blotting. Extracts in PBS of gonad (100 μg/ml), hepatopancreas (110 μg/ml), and hemolymph (110 μg/ml) from both male and female crabs were tested against the antibody in Ouchterlony plates (1% agar in PBS). Extracts of gonad, hepatopancreas, and hemolymph (100 μg/300 μl) were incubated at 4°C for 18 hours with 10 μl of rabbit serum. The mixtures were then centrifuged at 8,000 × *g* at 4°C for 10 min and the resulting protein pellets washed with ice cold PBS 4 times. Pellets of immunoprecipitated protein were solubilized in electrophoresis buffer containing sodium dodecyl sulfate and separated on a 7% polyacrylamide gel. This assay should recover the proteins that the antibody recognizes and precipitated from the crude extracts containing several different proteins. Extracts of gonads and hepatopancreas from females in vitellogenesis were separated on a 7% polyacrylamide gel in sodium dodecyl sulfate. The gel was then electroblotted onto nitrocellulose paper (Towbin *et al.*, 1979; Douglas and King, 1984). The rabbit serum was incubated with the filter paper blots for 2 h in PBS. A goat anti-rabbit IgG antibody with peroxidase conjugated to the antibody was then incubated with the blots in PBS (Kirkegaard and Perry, Gaithersburg, Maryland). The blots were then stained with 4-chloro-1-napthol (Sigma Chemical Co, St. Louis, Missouri) and H_2O_2. This western blotting procedure was used to determine if the rabbit serum bound selectively to the V_2 proteins contained in these extracts, when it was challenged with all the different proteins in the crude extracts.

Immunoassay

The immunoassay consisted of a 4-h *in vitro* incubation as described above. Tissues were then homogenized in extraction buffer. Proteins were extracted with 3 cycles of extraction buffer and ice cold 100% $(NH_4)_2SO_4$. The final protein pellet was resuspended in 600 μl of PBS. Aliquots (100 μl) were taken from this solution for determination of total protein and ^{14}C leucine incorporation. Fifty μl of the rabbit serum (25 μg protein) was added to the remaining 400 μl of each tissue homogenate and the mixture was incubated at 4°C for 18 hours. After incubation, the tissue homogenates were centrifuged at 8000 × *g* at 4°C for 15 minutes. The supernatant was carefully removed, and 100 μl aliquots were taken for determination of total protein and ^{14}C leucine incorporation. The pellet of immunoprecipitated protein was washed four times with ice cold PBS. The final protein pellet was solubilized in 500 μl of 1 *N* NaOH, and aliquots of 100 μl were taken for determination of total protein and ^{14}C leucine incorporation. Differences between treatment groups were tested for significance with a *t*-test (Sokal and Rohlf, 1969).

GIH purification

Samples of crude *P. setiferus* eyestalks (3000 μl) were applied to a Sephadex G-25 column (2.5 cm × 20 cm; 43 ml void volume determined with blue dextran) and eluted in 0.05 m*M* ammonium acetate, pH 6.3 at 3.0 ml/h. Fractions (1 ml) were monitored at 280 nm, collected, and then bioassayed for their ability to effect Vg synthesis *in vitro*. The retention factor, Rf, was calculated based on the formula: column void volume/fraction elution volume. Standard peptides (1 mg/ml) were used to calibrate the column for size estimation. Standard peptides used were: aprotinin, 6500 daltons; insulin (beta subunit), 3496 daltons; and met-enkephalin, 537 daltons. The effect of crude eyestalk extract or partially purified GIH was quantified in all *in vitro* assays using the formula:

$$\% \text{ Inhibition} = \frac{\text{Control DPM/mg} - \text{Test DPM/mg}}{\text{Control DPM/mg}} \times 100$$

A unit of GIH activity was defined as that amount of protein that produced a 20% inhibition of ^{14}C leucine incorporation *in vitro*. Based on a *t*-test for percentages, a 20% inhibition was statistically significant at $P < 0.05$ (Sokal and Rohlf, 1969). Using these calculations, a single shrimp eyestalk had about 160 units of activity.

Results

In vivo *studies*

Crude extracts of *P. setiferus* eyestalks blocked the anticipated increase in ovarian weight gain of eyestalk ablated crabs (Fig. 1). This was attributed to the blockade of vitellogenesis. The shrimp eyestalk extract blocked vitellogenesis even though the remainder of the crab's endocrine system was intact. The extract was potent, only 0.0005 eyestalk equivalents was required to produce a

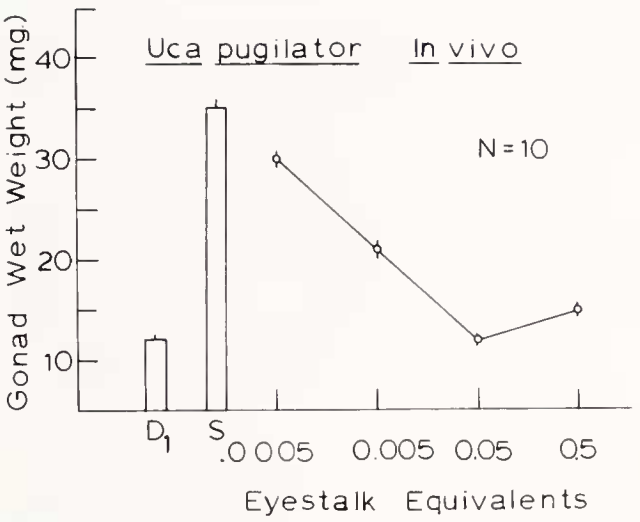

Figure 1. *In vivo* effects of crude shrimp eyestalk extract on ovarian growth of eyestalk ablated *Uca pugilator*. Extract dose was given in eyestalk equivalents, ovary wet weight was measured in milligrams. S = ovarian weight of crab injected with saline, D_1 = average ovarian wet weight for a group of crabs from which experimental crabs were selected at the start of the experiment. All values are the mean ± one standard error, n = 10, two replicates.

statistically significant inhibition of ovarian weight gain (*t*-test, $P < 0.05$).

The *in vivo* incorporation of ^{14}C leucine into proteins from the hepatopancreas, hemolymph, and ovaries was significantly higher in eyestalk-ablated crabs than intact crabs after 4 h (*t*-test, $P < 0.05$; Fig. 2). The average oocyte diameter of ovaries from eyestalk-ablated crabs (0.12 ± 0.01 mm) was twice the average oocyte diameter of ovaries in intact crabs (0.06 ± 0.01 mm). This may account for the significant differences in the incorporation of ^{14}C leucine, since the two groups of crabs had ovaries at different stages of vitellogenesis. Twenty-four hours after injection with ^{14}C leucine, incorporation of the labeled amino acid was about equal in the tissues from both intact and eyestalk-ablated crabs. Tissues from intact crabs had a significant increase in incorporation of labeled amino acid when incorporation time was increased from 4 to 24 hours (*t*-test, $P < 0.05$). The hepatopancreas and ovaries incorporated more labeled amino acid into proteins than the hemolymph in both groups of crabs.

In vitro *studies*

The time course of *in vitro* ^{14}C leucine incorporation was measured for muscle, hepatopancreas, and ovaries from intact and ablated crabs (Fig. 3). Incorporation of the labeled amino acid into muscle proteins was a control for non-specific binding of label and for the incorporation of label into proteins by a tissue that does not contain egg yolk proteins. There was a significant difference in the amount of label incorporated by ovary and hepatopancreas from intact crabs compared to these tissues from eyestalk-ablated crabs. The difference between the two groups was statistically significant at all measurements (*t*-test, $P < 0.01$–0.05). Initially, the ^{14}C leucine incorporation into proteins was about equal in the ovary and hepatopancreas from eyestalk ablated crabs. After 8 hours of incubation, the ovarian tissue continued to incorporate labeled amino acid into proteins; the hepatopancreas tissues did not increase their incorporation of labeled amino acid into proteins. The ovaries and hepatopancreas from intact crabs had about equal incorporation of labeled amino acid into proteins at 2 h and 8 h, though they had statistically different levels of incorporation of labeled amino acid into protein at 4 h (*t*-test, $P < 0.05$ at 4 h). After 8 h in the *in vitro* incubation, the tissues from the intact crabs followed the same pattern as the tissues from the eyestalk ablated crabs: the ovaries continued to incorporate label but the hepatopancreas did not increase incorporation of label. Variability in ^{14}C leucine incorporation into proteins increased with increased incubation times, as demonstrated by the increase in the standard deviations of these measurements (Fig. 3). This observation suggested that after 8 h of *in vitro* incubation the tissues were no longer uniformly active, and the assay would not produce an accurate pic-

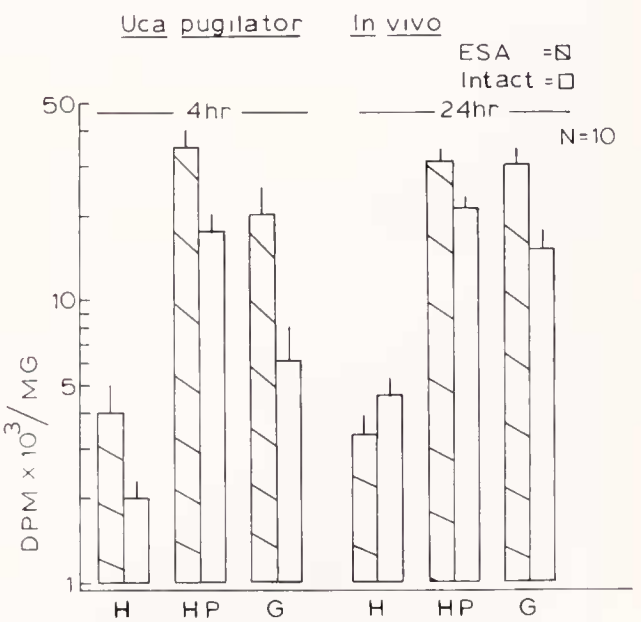

Figure 2. *In vivo* incorporation of ^{14}C leucine into proteins from the hemolymph (H), the hepatopancreas (HP), and ovary (G) of either intact (open bars) of eyestalk ablated (hatched bars) *Uca pugilator*. All values are the mean ± one standard error, n = 10 for each group, 2 replicates.

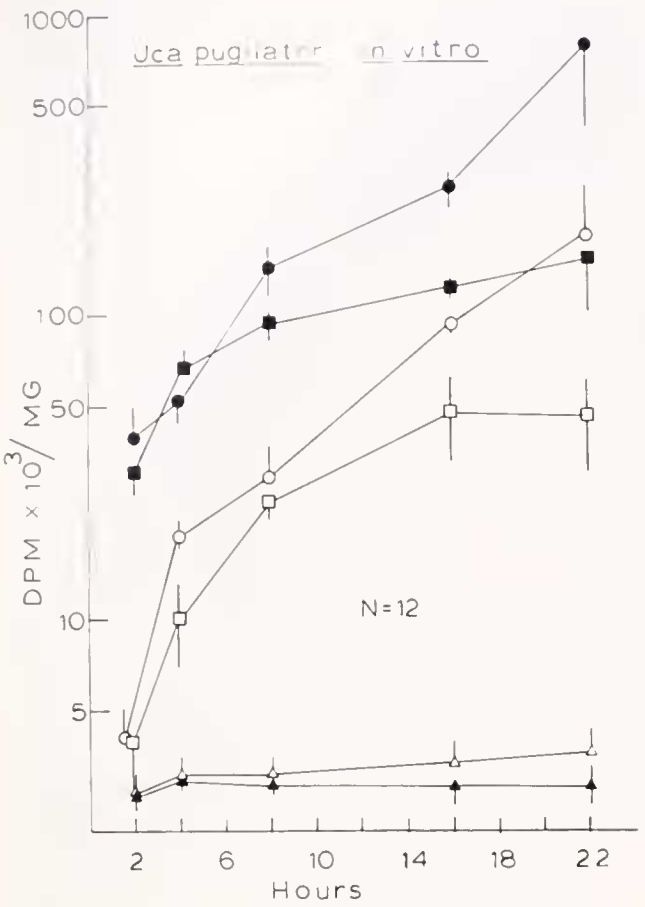

Figure 3. Time course of incorporation of ^{14}C leucine into proteins from muscle (triangles), ovaries (circles), and hepatopancreas (squares) from either intact crabs (open symbols) or eyestalk-ablated crabs (filled symbols) measured *in vitro*. All values are means ± one standard error, n = 12 for each group, 2 replicates.

ture of protein synthesis. A 4-h incubation time was used for all subsequent assays.

Crude shrimp eyestalk extracts were tested for their ability to affect *in vitro* protein synthesis in the ovaries from intact and eyestalk-ablated crabs (Fig. 4). The threshold for statistically significant inhibition of protein synthesis was the same regardless of the origin of the ovarian tissues (0.006 ESE, *t*-test for two percentages, $P < 0.05$). Maximum inhibition of the protein synthesis was produced by 0.05 ESE in ovaries from eyestalk ablated crabs. Protein synthesis in ovaries from intact crabs was maximally inhibited by 0.012 ESE. Ovaries from the intact crabs were more sensitive to the eyestalk extract than ovaries from ablated crabs. A dose of 0.012 ESE produced a significantly greater inhibition in the ovaries from intact crabs than it did in the ovaries from eyestalk ablated crabs (*t*-test for two percentages, $P < 0.05$). The highest dose tested, 0.05 ESE produced less, not more, inhibition of protein synthesis than a lower dose. This suggests that the crude extract probably contains many factors, perhaps even a factor that can increase protein synthesis *in vitro* (Charniaux-Cotton, 1985).

Vitellogenin purification and antibody characterization

The partially purified extract of ovaries from *Uca pugilator* was dominated by the two distinct bands of protein characteristic of the egg yolk proteins (Eastman-Reks and Fingerman, 1984; Fig. 5A, B). The molecular weights of the two groups of egg yolk proteins were 103,000 ± 1000 daltons, V_1, and 81,000 ± 1000 daltons, V_2 (n = 12 measurements, Fig. 5A, B). These bands of protein in the polyacrylamide gels clearly represent two classes of proteins, which each may contain several yet unresolved distinct polypeptides. The hepatopancreas extracts contained only the V_2 group of egg yolk proteins (n = 8; Fig. 5A, B). Both V_1 and V_2 are the dominant proteins of the egg yolk, they stain positive for both lipid and sugars, and they contain the purple pigment of the egg yolk. Thus, these proteins, V_1 and V_2 fulfill the criteria established for vitellogenin (Eastman-Reks and Fingerman, 1984; Wallace *et al.*, 1967).

The antiserum (#1790-12-4) to the V_2 protein group

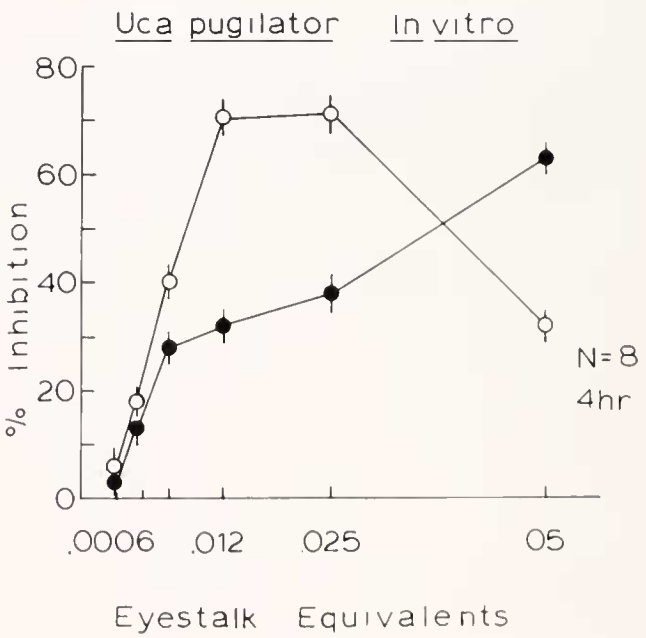

Figure 4. The *in vitro* inhibition of incorporation of ^{14}C leucine into ovarian proteins by crude shrimp eyestalk extract. Ovarian tissues from intact crabs (open circles) or eyestalk ablated crabs (filled circles) were tested. All values are means ± one standard error, n = 8 for each group, 2 replicates. An extract of shrimp tail muscle was used for a control for eyestalk extracts. Control injections were adjusted by dilution with crab saline to equal the total protein concentration of the injections of eyestalk extract. Percent inhibition was calculated as described in Materials and Methods.

reacted with crude extracts from female crab gonads, hepatopancreas, and hemolymph in Ouchterlony plates. The antiserum did not produce any reaction with similar extracts of tissue from male crabs. Therefore the antiserum was specific for female proteins (data not shown). The antiserum precipitated both V_1 and V_2 proteins from ovary extracts, but only V_2 proteins from hepatopancreas extracts (Fig. 5A). Thus when the antiserum was presented with crude tissue extracts containing many different proteins and some breakdown products of large proteins, the antiserum only precipitated the V_1 and V_2 proteins. The western blot of proteins from crude tissue extracts showed that the antiserum selectively bound to only the V_2 proteins in the hepatopancreas (Fig. 5C). The antiserum did bind to some low molecular weight proteins other than V_2 in the ovarian homogenate. The antiserum had low affinity for any of the other proteins known to be in these crude extracts (Fig. 5A–C). Thus this antiserum (#1790-12-4) was specific to female proteins, and relatively specific for V_1 and V_2 proteins from tissue extracts. The immunoprecipitation of both V_1 and V_2 from the ovarian tissue extracts suggests that these two proteins may be linked in the ovary.

The antibody was used to measure *in vitro* Vg synthesis in homogenates of ovaries, hepatopancreas and hemolymph (Table I). The ovary had more immunoprecipitatable protein than either the hepatopancreas or the hemolymph, consistent with the role of the ovary as a yolk storage site. However, the ^{14}C leucine content of the immunoprecipitated Vg was similar in both the ovary and hemolymph samples, suggesting that both ovary and hemolymph can produce new egg yolk proteins. Recovery of all the proteins and radioactive labeled amino acid was near 90% for these assays. Some label and protein was lost due to the procedures used. Though the amount of immunoprecipitated Vg was less in the hepatopancreas than the ovary, the Vg in the hepatopancreas had about three times the ^{14}C leucine incorporated into Vg than the ovary. This is consistent with the hepatopancreas as a site of Vg production but not Vg storage. Ovary, hepatopancreas, and hemolymph can incorporate ^{14}C leucine into new egg yolk proteins, the hepatopancreas incorporates much more than the other groups, whereas the ovary seems to retain more Vg than either the hepatopancreas and the hemolymph.

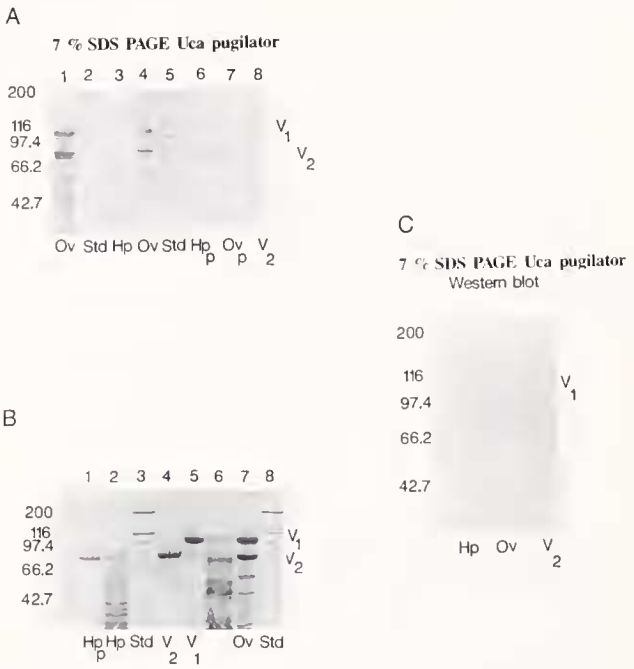

Figure 5. Isolation of egg yolk proteins from ovaries (Ov) and hepatopancreas (Hp) from the tissues of *Uca pugilator*. A. Proteins from crude extracts of ovary and hepatopancreas. Immunoprecipitates from these crude extracts of ovary (Ov_p) and hepatopancreas (Hp_p) are compared to the complete extracts and a sample of partially purified V_2 (V_2). In the sample of ovarian proteins the antibody precipitated both V_1 and V_2, but only V_2 was precipitated from the sample of hepatopancreas. The location of the molecular weight standards are labeled on the left side in daltons $\times 10^3$ B. Isolation of egg yolk proteins. Lane 7 contains a crude ovarian homogenate. Lane 6 contains the fraction with purple color from the G-200 column. Lanes 5 and 4 contain the proteins eluted from the preparative polyacrylamide gels, labeled V_1 and V_2 respectively. Lane 2 contains a crude extract of hepatopancreas, while lane 1 is the immunoprecipitated protein from this hepatopancreas homogenate. C. Western blot of a 7% polyacrylamide gel. Samples of crude ovarian and hepatopancreas extract were separated in a polyacrylamide gel, a sample of V_2 was run in the third lane. The polyclonal antibody to V_2 bound to a single band in the hepatopancreas sample, and to three bands in the ovarian sample. The antibody did not bind to V_1, though it was present in the ovarian sample.

Partial purification of GIH

Fractions from crude shrimp eyestalk extract were tested for their ability to inhibit *in vitro* ovarian protein synthesis. The values for immunoprecipitated Vg are given in Figure 6 and some values for non-specific inhibition are reported in Table II. Eighty-six percent of the inhibitory activity applied to the column was recovered in the 35 fractions that were tested (5 replicates). Three peaks of inhibitory activity were consistently resolved (fraction #29, Rf = 0.48; fraction #35, Rf = 0.41; and fraction 64, Rf = 0.23, 5 replicates). These fractions had 64% of all the inhibitory activity applied to the column. All three fractions had about the same specific activity (2200 ± 300 units/mg; 5 replicates), fraction 29 at Rf = 0.48 had the most protein (3 μg ± 0.7 μg; 5 replicates). The size of proteins eluting at fraction #29, Rf = 0.48 was estimated to be 3,300 ± 500 (5 replicates). This is a smaller size estimate for GIH that the GIH previously isolated from the lobster, *Panulirus argus*, (Quackenbush and Herrnkind, 1983). Based on the bioassay, the column chromatography produced a 37-fold purifica-

Table I

Immunoprecipitation [illegible] *stacs of eyestalk ablated crabs* Uca p[illegible]

Sample	Proteins (mg)		
	Ovary	Hepatopancreas	Hemolymph
Crude Extract	0.51 ± 0.09	0.55 ± 0.06	0.50 ± 0.01
Pellet	0.061 ± 0.04	0.045 ± 0.01	0.014 ± 0.01
Supernatant	0.421 ± 0.05	0.401 ± 0.02	0.390 ± 0.01

Sample	DPM/MG/Hour		
	Ovary	Hepatopancreas	Hemolymph
Crude Extract	5,611 ± 1,065	8,536 ± 1,425	1,448 ± 123
Pellet	865 ± 181	2,977 ± 755	792 ± 71
Supernatant	2,973 ± 1,208	3,803 ± 567	778 ± 79

All values are means ± one standard deviation, n = 12 for all cases, 2 replicates. Crude extract is the initial tissue homogenate, Pellet is the protein precipitated by the antibody to vitellogenin, Supernatant is the protein not precipitated by the antibody. Recovery of both label and protein was between 80–90% for these assays.

tion from the crude material (fraction #29, Rf = 0.48). Fraction #29 inhibited Vg ^{14}C leucine incorporation. It was specific to Vg proteins. Fraction #29 had no significant effect on ^{14}C leucine incorporation into proteins other than Vg, represented by the supernatant in the immunoassay (Table II). Both the crude starting material and material from the column void volume inhibited ^{14}C leucine incorporation into both Vg and non-Vg proteins, demonstrating non-specific protein synthesis inhibition (Table II, Fig. 6). Based on the size estimate for fraction #29 (3,300 ± 500 daltons) the material in this fraction was biologically active at 1.8×10^{-9} *M* protein.

Discussion

The primary source of crustacean egg yolk proteins was first suggested to be extra-ovarian (Wallace *et al.*, 1967). This hypothesis was consistent with the demonstrations of egg yolk protein synthesis in insects and vertebrates. However, evidence from histological studies of developing crustacean ovaries suggested that the ovaries were capable of producing proteins (Beams and Kessel, 1963; Ganion and Kessel, 1972; Wolin *et al.*, 1973; Schade and Schivers, 1980). Direct demonstration of protein synthesis by ovarian tissue supported the new view that the ovarian contribution to overall egg yolk protein synthesis was significant. The relatively slow rate of ovarian protein synthesis in isolated ovarian tissue supported the argument that extra-ovarian tissue also contributed to egg yolk protein synthesis (Lui and O'Connor, 1976, 1977; Eastman-Reks and Fingerman, 1985).

Ovarian egg yolk protein synthesis does not preclude extra-ovarian egg yolk protein synthesis. The unstated assumption in previous work was that egg yolk proteins in crustaceans were produced exclusively in a single tissue, as in insects and vertebrates. Insects produce vitellogenin exclusively in the fat body. A similar pattern was expected in arthropod relatives, the crustaceans. In the isopod, *Idotea bathica basteri*, and the amphipod, *Orchestia gammarella*, egg yolk proteins are produced in a subepidermal adipose tissue, the fat body (Blanchet-Tournier, 1982; Souty and Picaud, 1984). However, vitellogenin is also made in the ovaries of the crab *Pachygrapsus crassipes* (Lui and O'Connor, 1977) and in the hemocytes of the crab, *Callinectes sapidus* (Kerr, 1969). The subepidermal adipose tissues of a shrimp, *Parapenaeus longirostris*, and the hepatopancreas of the crabs, *Carcinus maenus* and *Libinia emarginata*, contain immunoreactive vitellin (Paulus and Laufer, 1987; Tom *et al.*, 1987). Together, these recent reports suggest several new sites for egg yolk protein synthesis.

In our study, immunoreactive Vg was found in the hemolymph, hepatopancreas, and ovaries of the crab, *Uca pugilator*. Each tissue was evaluated for its capacity to incorporate labeled amino acids into proteins and Vg

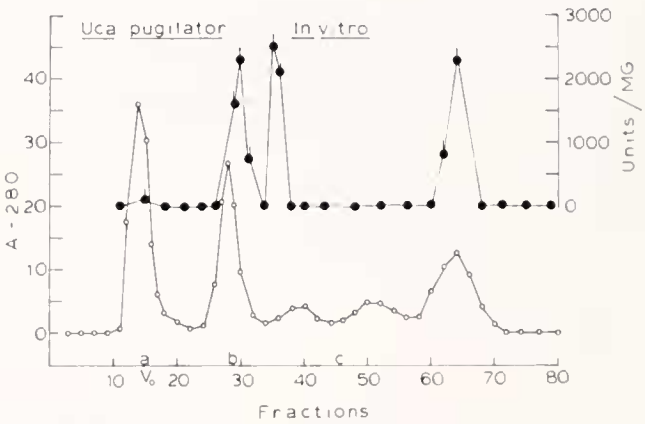

Figure 6. Immunoassay of the fractions of crude shrimp eyestalk extract after separation on G-25 Sephadex. Filled circles are the specific activity (Inhibition units/mg eyestalk extract protein) of the fractions for the inhibition of ^{14}C leucine incorporation into ovarian egg yolk proteins. Open circles are the absorbance (280 nm) of the fractions as they were eluted from the column. The column void volume is indicated by a V. The elution volumes of protein standards used to calibrate the G-25 Sephadex column are indicated by a = aprotinin, b = insulin beta chain, and c = met-enkephalin. The immunoassay values are means ± one standard error for n = 8 for each fraction, 4 replicates. Extracts of shrimp tail muscle were adjusted with crab saline to equal the protein concentrations of the various column fractions of shrimp eyestalk. These tail muscle extracts served as the controls in the *in vitro* assay. Percent inhibition of ^{14}C leucine incorporation into crab egg yolk proteins was calculated as described in Materials and Methods. Every other fraction was bioassayed, but some fractions (with no activity) were not plotted for clarity.

Table II

*Bioassay of shrimp (*Penaeus setiferus*) eyestalk extract on* in vitro *vitellogenin incorporation of labeled leucine in isolated ovarian fragments from the fiddler crab (*Uca pugilator*)*

	% Inhibition of protein incorporation of labeled leucine		
G-25 Fraction #	Pellet	Supernatant	
Crude material	64%*	22%*	
# 15 (void volume)	64%*	22%*	
# 22	6%	4%	
# 29	64%*	6%	Rf = 0.48
# 31	48%*	14%	
# 35	58%*	12%	
# 42	7%	10%	
# 52	5%	6%	
# 64	28%*	12%	
# 70	3%	5%	

All values are means, n = 8 for each fraction from 4 replicates. An * indicates a statistically significant difference between the fraction tested and a muscle extract control (t test for two percentages, $P < 0.05$). One unit of GIH activity produces a 20% inhibition of protein incorporation of labeled leucine, by definition. Crude material is the initial eyestalk extract; all test does were adjusted with saline to a protein concentration of 10 μg/ml. Fraction numbers in this table correspond with fraction numbers in Figure 6. Fractions were selected from a complete data set (35 fractions) as representative of the observations.

in vitro. Comparisons of protein synthesis in the tissues from *Uca pugilator* to other crustaceans is limited by the important variations in the reported methods, procedures, and other variables. Ovarian incorporation of labeled amino acids into egg yolk proteins from *Procambarus* sp. (477 DPM/mg/h) and *Pachygrapsus crassipes* (966 DPM/mg/h) were suggested to be too low to account for complete vitellogenesis (Lui and O'Connor, 1977). Incorporation of labeled amino acids into egg yolk proteins in the hepatopancreas of the fiddler crab (2927 DPM/mg/h) was more than three times the incorporation of labeled amino acids into egg yolk proteins in either the ovarian tissue or the hemolymph. This significant difference (*t*-test, $P < 0.05$) suggests that the hepatopancreas in the fiddler crab can contribute to overall egg yolk protein production.

The demonstrated role of the crustacean hepatopancreas is the synthesis and secretion of digestive enzymes (Gibson and Barker, 1979). This tissue is also a major site of lipid storage and carbohydrate metabolism (Chang and O'Connor, 1983; Sedlmeier, 1985). In the lobster, *Homarus americanus*, the hepatopancreas is the principal tissue source of hemocyanin synthesis (Senkbeil and Wriston, 1981). Eyestalk factors can affect lipid metabolism, protein synthesis, enzyme synthesis, and ribonucleic acid synthesis in the crustacean hepatopancreas (Fingerman *et al.*, 1967; O'Connor and Gilbert, 1968; Gorell and Gilbert, 1971; Bollenbacher *et al.*, 1972; Wormhoudt, 1974; Momin and Rangneker, 1975). In our study, eyestalk ablation significantly increased both *in vivo* and *in vitro* incorporation of labeled amino acids into proteins of the hepatopancreas. Crude extracts of eyestalks decreased protein synthesis in the hepatopancreas of the crayfish, *Orconectes virilis*, but increased ribonucleic acid synthesis in the hepatopancreas of the crayfish, *Procambarus clarkii* (Fingerman *et al.*, 1967; Gorell and Gilbert, 1971). Both protein synthesis and egg yolk protein synthesis in ovaries and hepatopancreas appear to be affected by the eyestalk endocrine system in crustaceans. The coordination of egg yolk protein synthesis in several tissues by the eyestalk endocrine system would be one mechanism to optimize the energy investment required for the production of many yolk-laden eggs.

Insects produce egg yolk proteins exclusively in the fat body (Downer and Laufer, 1983). Fiddler crabs can make egg yolk proteins in at least three sites: ovaries, hepatopancreas, and hemolymph. Other crustaceans appear to produce egg yolk proteins from several tissues as well (Blanchet-Tournier, 1982; Charniaux-Cotton, 1985; Fingerman, 1987). One hypothesis for these fundamental differences among the arthropods may be linked to the differences in life histories. Pterygote insects do not molt as adults, whereas most adult crustaceans continue to molt. Molting is a physiologically demanding process requiring extensive protein synthesis and lipid metabolism (Chang and O'Connor, 1983; Skinner, 1985). The repetitive egg yolk production of adult crustaceans which live for several years is an equally demanding physiological process. The mature ovary of a fiddler crab is 4–6% of the total body wet weight; the ovary contains about 30–40 mg of egg yolk proteins (Webb, 1977). During the reproductive period in the summer, a single female crab will produce two broods of several thousand eggs (Webb, 1977; Christy, 1978). The eyestalk endocrine system is capable of regulating both molting and ovarian development with inhibitory factors so that these two process do not occur simultaneously (Webb, 1977; Adiyodi, 1985). The established need for the synchronization of egg release or egg hatching to systematic environmental variation further constrains the production of mature oocytes (Hartnoll, 1969; Christy, 1982). Fiddler crabs precisely time the release of larvae to optimize their survival (Bergin, 1981; Christy, 1982). Both physiological and physical limitations may require that the massive egg yolk protein synthesis be completed quickly. Therefore, using several sites to synthesize egg yolk proteins may be one strategy to maximize both somatic growth and reproductive output within these constraints. The diversity of crustacean life histories and the habitats they exploit make this a testable hypothesis (Christy, 1982; Hartnoll, 1969).

The partially purified GIH inhibited egg yolk protein synthesis directly in the isolated ovaries. Use of antibody to Vg allows the specific measurement of egg yolk protein production. This Vg-specific assay is a significant improvement over previous assays for GIH which were based on overall protein synthesis or simply ovarian wet weight changes (Bomirski *et al.*, 1981; Quackenbush and Herrnkind, 1983; Eastman-Reks and Fingerman, 1984; Charniaux-Cotton, 1985). The development of a bioassay for GIH or any inhibitory hormone requires adequate controls to detect the potential toxic effects of non-specific agents that may be present in crude extracts (Channing *et al.*, 1985). Bomirski *et al.* (1981) found toxic fractions in their extraction of GIH from the crab, *Cancer magister*. The toxicity was attributed to the large amounts of protein they injected into their bioassay animals. They required a 0.5 ESE dose to produce a measurable response in the shrimp, *Crangon crangon*. The material we extracted from the shrimp eyestalks required only a 0.005 ESE dose *in vivo* and a 0.006 ESE dose *in vitro* to produce statistically significant inhibitory responses in *Uca pugilator*. The potency of the crude extract permitted much less total protein in our assays for GIH than previous assays required. This may have helped us avoid a non-specific protein-induced toxic effect of crude extracts. The partially purified GIH blocked incorporation of labeled amino acids into Vg, did not affect the incorporation of labeled amino acids into other proteins. Crude extract and a few fractions near the void volume of the column did have the ability to block labeled amino acid incorporation into both Vg and other proteins. This non-specific inhibition of incorporation of labeled amino acids serves as a control for non-specific effects in our *in vitro* assay system. Thus, with the *in vitro* assay procedure, we can measure specific inhibition of labeled amino acid incorporation into Vg, as well as the non-specific inhibition of incorporation of labeled amino acids into proteins that are not Vg. This *in vitro* procedure can now be used in the further characterization of eyestalk neurohormones.

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